CARD9-Expressing Microglia Promote IL-1β and CXCL1-dependent Neutrophil Recruitment to the Fungal-Infected Central Nervous System

Rebecca A. Drummond1†, Muthulekha Swamydas1#, Vasileios Oikonomou1#, Bing Zhai2#, Ivy Dambuza3, Brian C. Schaefer4, Andrea C. Bohrer5, Katrin D. Mayer-Barber5, Sergio A. Lira6, Yoichiro Iwakura7, Scott G. Filler8, Gordon D. Brown3, Bernhard Hube9,10, Julian R. Naglik11, Tobias M. Hohl2, Michail S. Lionakis1*

1Fungal Pathogenesis Section, Laboratory of Clinical Immunology and Microbiology (LCIM), National Institute of Allergy & Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, MD, USA
2Infectious Disease Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY, USA
3Medical Research Council Centre for Medical Mycology at the University of Aberdeen, Aberdeen Fungal Group, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, UK
4Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD
5Inflammation and Innate Immunity Unit, LCIM, NIAID, NIH, Bethesda, MD, USA
6Immunology Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
7Research Institute of Biomedical Sciences, Tokyo University of Science, Tokyo, Japan
8Division of Infectious Diseases, Department of Medicine, Los Angeles Biomedical Research Institute at Harbor—UCLA, Torrance, CA, 90502
9Department of Microbial Pathogenicity Mechanisms; Leibniz Institute for Natural Product Research and Infection Biology; Hans Knöll Institute Jena; Jena, Germany
10Friedrich Schiller University, Jena, Germany
11Mucosal and Salivary Biology Division, King’s College London Dental Institute, King's College London, London, United Kingdom
12†Current address: Institute of Immunology & Immunotherapy, Institute of Microbiology & Infection, University of Birmingham, Birmingham B15 2TT, UK
13*These authors contributed equally to this work
14*Correspondence: r.drummond@bham.ac.uk; lionakism@niaid.nih.gov
Abstract

The C-type lectin receptor/Syk adaptor CARD9 facilitates protective antifungal immunity within the central nervous system (CNS), as human CARD9-deficiency causes fungal-specific CNS-targeted infection susceptibility. We previously showed that CARD9 is required for neutrophil recruitment to the fungal-infected CNS, which mediates fungal clearance. Here, we investigated host and pathogen factors that promote protective neutrophil recruitment during Candida albicans CNS invasion and examined their dependence on CARD9 for in vivo induction. We show that IL-1β is essential for CNS antifungal immunity by driving CXCL1 production, which recruits CXCR2-expressing neutrophils. Neutrophil-recruiting IL-1β and CXCL1 production is induced in microglia by the fungal-secreted peptide toxin Candidalysin, in a p38-cFos-dependent manner. Importantly, microglia rely on CARD9 for production of IL-1β, via both pro-IL-1β transcriptional regulation and inflammasome activation, and of CXCL1 in the fungal-infected CNS, and we show that microglia-specific CARD9 deletion impairs IL-1β and CXCL1 production and neutrophil recruitment, and increases CNS fungal proliferation. Our data reveals an intricate network of host-pathogen interactions that promotes CNS antifungal immunity and provides novel mechanistic insights into how human CARD9-deficiency causes CNS fungal disease.
**Introduction**

The CNS can be invaded by microorganisms during the course of systemic infections, yet our understanding of the regulation of CNS-specific anti-microbial immune responses is poor. This is particularly true for meningitis and CNS parenchymal infection by pathogenic fungi, which present unmet clinical challenges since they require a high degree of suspicion for diagnosis and treatment options are limited. Indeed, fungal CNS infections have unacceptably high mortality rates (>50%) and remain the most lethal form of disease for most fungal species\(^1, 2, 3\).

Susceptibility to fungal CNS invasion is enhanced by fungal-specific risk factors. For example, cryptococcal meningitis is associated with HIV infection/AIDS or idiopathic CD4 lymphocytopenia\(^4, 5\), while CNS invasion by *Candida albicans* and *Aspergillus fumigatus* occur in immunosuppressed patients with prolonged neutropenia and/or corticosteroid use\(^6\). Novel iatrogenic risk factors for CNS cryptococcosis and aspergillosis have recently emerged in hematology patients receiving the Bruton’s tyrosine kinase inhibitor ibrutinib\(^7, 8\). However, the most striking human risk factor for selective susceptibility to CNS fungal infection (most often by *C. albicans*) to date is inherited deficiency of the C-type lectin receptor (CLR)/Syk adaptor CARD9.

CARD9 is a signaling adaptor protein downstream of members of the CLR superfamily, including Dectin-1, Dectin-2, Dectin-3 and Mincle\(^9\). CLRs are important PRRs for fungal recognition and activation of anti-fungal immune responses via the initiation of intracellular signaling cascades. Syk kinase is first recruited to phosphorylated ITAM sequences on the
intracellular tails of CLRs or their signaling partner chain (e.g. FcRγ), to form the CBM signalosome, containing CARD9, BCL10 and MALT1. This tri-molecular complex activates multiple downstream effectors including the transcription factor NFκB, the NLRP3 inflammasome and MAPK signaling.

CARD9-deficient patients are unable to respond selectively to fungal stimuli, and manifest profound and specific susceptibility to fungal diseases, predominantly in the CNS caused by C. albicans, the most common human fungal pathogen. We recently showed that CARD9-deficiency in humans and mice confers a fungal- and brain-specific defect in neutrophil accumulation resulting in CNS-neutropenia. This lack of neutrophil recruitment is detrimental for control of CNS fungal invasion, because neutrophils are the most important immune cells for controlling systemic C. albicans infections and an early neutrophil influx is required for preventing C. albicans infection within the CNS. However, the CNS cellular and molecular cues that promote protective neutrophil recruitment into the C. albicans-infected CNS and their dependence on functional CARD9 for induction in vivo remain unknown.

Herein, we systematically investigated the molecular host and pathogen factors that promote early protective neutrophil influx into the C. albicans-infected CNS and confer host resistance against fungal CNS invasion with an aim to better understand the pathogenesis of human CARD9-deficiency. For this, we examined 20 mouse strains lacking various CARD9-coupled PRRs, signaling proteins, cytokines, chemokines and their receptors. We uncover an intricate immune pathway by which the C. albicans-secreted peptide toxin Candidalysin engages microglia, the resident phagocytes of the CNS, to produce IL-1β and CXCL1 for protective
recruitment of CXCR2-expressing neutrophils. Importantly, we show that IL-1β and CXCL1 production by microglia depends on CARD9 in the *C. albicans*-infected CNS and demonstrate that specific deletion of microglial CARD9 results in impaired neutrophil recruitment to the *C. albicans*-infected CNS and CNS-specific inability to control fungal proliferation *in vivo*. Collectively, our data unveil complex host-pathogen interactions occurring within the CNS to promote protective neutrophil recruitment during fungal invasion and reveal the mechanism that underlies CNS susceptibility to fungal invasion in CARD9-deficiency.
Results

CARD9-coupled CLRs Functionally Compensate to Protect the Brain from Fungal Invasion

As shown previously in mice and humans, CARD9 is an essential component of the protective immune response to *C. albicans* in the brain, principally through the recruitment of neutrophils early post-infection (Fig. 1a). However, the CARD9-dependent cellular and molecular factors that mediate protective neutrophil recruitment in the *C. albicans*-infected brain remain unknown. Therefore, we first decided to investigate the relative contribution of known CARD9-coupled CLRs, which are expressed by brain-resident microglia (Supplementary Fig. 1), in mediating protective early neutrophil recruitment to the brain. We systemically infected mice deficient in Dectin-1 (*Clec7a*<sup>−/−</sup>), Dectin-2 (*Clec4n<sup>−/−</sup>), Dectin-3 (*Clec4d<sup>−/−</sup>) and Mincle (*Clec4e<sup>−/−</sup>) and measured accumulation of neutrophils in the brain at 24 hours post-infection (Supplementary Fig. 2). We chose this time-point since we have previously found that this is the peak of the neutrophil response in wild-type (WT) animals and that depletion of neutrophils at this time-point confers increased susceptibility to brain fungal invasion<sup>13</sup>.

We found that animals individually deficient in CARD9-coupled CLRs recruited neutrophils to the *C. albicans*-infected brain normally (Fig. 1b). Despite the normal early neutrophil influx, we did observe increases in fungal brain burdens at 72 hours post-infection in mice deficient in Dectin-1 or Dectin-2 but not in Dectin-3 or Mincle (Fig. 1c), suggesting that Dectin-1 and Dectin-2 employ mechanisms other than neutrophil recruitment to protect the brain against fungal proliferation, possibly via promoting neutrophil, recruited monocyte and/or resident
microglia fungal phagocytosis and/or killing. Indeed, we found significant reductions in the phagocytosis of GFP-expressing *C. albicans* within brain-infiltrating neutrophils in the Dectin-1 and Dectin-2-deficient animals (Supplementary Fig. 3), consistent with similar prior findings in Dectin-1-deficient bone marrow neutrophils and Dectin-2-deficient peritoneal neutrophils\textsuperscript{16, 17}. Further study will be needed to clarify the organ- and cellular-specific functions of these CLRs.

To activate CARD9-dependent signaling, phosphorylation occurs on the ITAM sequence found within the intracellular tail of Dectin-1 or the FcR\(\gamma\) chain, which Dectin-2, Dectin-3 and Mincle associate with to initiate intracellular signaling cascades\textsuperscript{18}. Therefore, we decided to assess whether deletion of all four CARD9-coupled CLRs affected the neutrophil response in the *C. albicans*-infected brain. We used mice doubly-deficient in Dectin-1 and FcR\(\gamma\) (Clec7a\textsuperscript{\textasciitilde}/\textasciitilde Fcerg1\textsuperscript{\textasciitilde}/\textasciitilde) and found that loss of both Dectin-1 and the FcR\(\gamma\)-coupled CLRs led to a phenotype that mimicked what we observed in Card9-deficient mice (Fig. 1a), in that there was a significant reduction in neutrophil recruitment and a corresponding increase in fungal burdens in the brain (Fig. 1b,c). Taken together, our data indicate that CARD9-coupled CLRs are able to functionally compensate to mediate neutrophil recruitment-dependent protection against *C. albicans* invasion of the brain.

**MALT1 is required for Defense against CNS Candidiasis**

The CBM signalosome CARD9-MALT1-BCL10 is necessary for the transduction of intracellular signals in response to fungi\textsuperscript{9}. Human deficiency of either MALT1 or BCL10 has been described in a small number of patients\textsuperscript{19, 20, 21, 22}. Deficiencies in these molecules profoundly affect both the innate and adaptive immune system arms, and as a result many of these patients die in
early childhood from overwhelming bacterial and viral infections. Human MALT1 deficiency has been additionally associated with the development of mucosal candidiasis, suggesting that anti-fungal immunity is impaired in these patients. However, whether MALT1 deficiency also predisposes to invasive brain-targeted candidiasis is unknown. To test this, we infected Malt1-deficient mice and assessed control of *C. albicans* growth in the brain as before. We found that *Malt1*−/− animals recruited neutrophils to the brain early after infection similarly to WT, however these animals exhibited uncontrolled fungal growth in the brain at 72 hours post-infection (*Fig. 1d*). These data indicate that MALT1 is critical for protective immune responses against *C. albicans* in the brain; however, the MALT1-dependent mechanisms of immunity operating in this tissue are independent of early neutrophil recruitment, unlike CARD9.

Importantly, MALT1-deficient patients may be at risk for CNS fungal disease. Therefore, a high index of suspicion is required in these patients to identify potential early signs of such infections, and consideration for initiation of antifungal prophylaxis is warranted in identified MALT1-deficient individuals to prevent fungal disease.

**IL-1β/IL-1R Signaling Promotes Early Neutrophil Recruitment to the Fungal-Infected Brain**

To gain insight into the local CNS tissue molecular cues that promote protective neutrophil recruitment in the *C. albicans*-infected CNS *in vivo*, we next examined the role of key cytokine and chemokine circuits by using a series of gene-deficient mice. We began by infecting IL-1 receptor (IL-1R)-deficient mice, because production of IL-1β by human peripheral blood mononuclear cells upon fungal-specific stimulation depends on CARD9. The IL-1R has been previously shown to promote neutrophil recruitment in a variety of settings, including the
oral mucosa following *C. albicans* mucosal infection\textsuperscript{25}, the lungs during *A. fumigatus* pulmonary infection\textsuperscript{26, 27}, and the brain following infection with group B *Streptococcus*\textsuperscript{28}; yet, whether IL-1R mediates neutrophil recruitment during *C. albicans* CNS invasion is unknown. Upon *C. albicans* challenge, we found that IL-1R-deficient mice phenocopied the Card9-deficient mice, with a loss of early neutrophil recruitment to the brain and accompanying increase in fungal brain burdens (Fig. 2a,b). Consonant with the dependence on IL-1R for protective neutrophil recruitment in the *C. albicans*-infected brain, loss of MyD88, the signaling adaptor protein downstream of IL-1R, resulted in a similar severely compromised ability to recruit neutrophils in the infected brain and to control fungal proliferation (Fig. 2a,b).

Given that IL-1R/MyD88 signaling was critical, we next assessed which IL-1R ligands were important for activating this signaling pathway and driving protection in the brain. To this end, we infected mice deficient in IL-1\(\alpha\), IL-1\(\beta\) or both. We found that mice lacking IL-1\(\alpha\) had a small reduction in neutrophil numbers and a slight increase in fungal burden in the brain at 24 hours post-infection (Fig. 2a,b). However, the lack of IL-1\(\alpha\) appeared to be compensated by the presence of IL-1\(\beta\), since *Il1a*\(^{-/-}\) animals recovered and were able to control fungal brain infection similar to WT by 72 hours post-infection and thus did not exhibit the inexorable fungal proliferation seen in IL-1R-deficient animals (Fig. 2b). In keeping with the critical contribution of IL-1\(\beta\), mice deficient in IL-1\(\beta\), or both IL-1\(\alpha\)/IL-1\(\beta\), had significant reductions in neutrophil accumulation and were highly susceptible for brain invasion, reaching over a million CFU per gram of brain tissue by 72 hours post-infection, similar to IL-1R-deficient animals (Fig. 2a,b). Therefore, IL-1\(\beta\) is a critical mediator of neutrophil recruitment to promote control of *C. albicans* infection in the brain.
Neutrophil Recruitment to the Fungal-Infected Brain Depends on the CXCL1/CXCR2 Chemoattractant Axis

Tissue-specific inflammatory signals, including those downstream of IL-1R, orchestrate the production of local mediators that recruit immune cells to the site of infection. Previously, we had shown that the CNS-neutropenia observed in mouse and human CARD9-deficiency is not caused by a neutrophil-intrinsic chemotaxis defect. Instead, human CARD9-deficient C. albicans-infected cerebrospinal fluid (CSF) did not promote chemotaxis of WT human neutrophils, as opposed to normal chemotaxis elicited by human CARD9-sufficient C. albicans-infected CSF. These data collectively indicate that the neutrophil trafficking defect is caused by insufficient production of soluble mediators in the CARD9-deficient infected CNS tissue. However, which among the several chemoattractants and their receptors promote(s) protective neutrophil recruitment to the C. albicans-infected CNS is unknown.

CARD9 has been shown to drive production of the CXCR2 ligands CXCL1 and CXCL2 in an autoantibody-induced arthritis models of inflammation and in the skin during murine subcutaneous phaeohyphomycosis. During systemic C. albicans infection, CCR1 promotes neutrophil accumulation to the infected kidney and is partly responsible for driving immune-related renal destruction, the leukotriene B4 (LTB4) receptor LTB4R1 promotes neutrophil accumulation to the infected lungs and is responsible for driving capillaritis and pulmonary tissue damage, and CXCR1 promotes neutrophil-dependent fungal killing in the C. albicans-infected kidney and in human cells. However, the role of these receptors in CNS anti-
Candida immunity is unknown. In addition, the role of CXCR2 and fMet-Leu-Phe (fMLP) receptor FPR1 have not been previously examined in the context of CNS antifungal immunity.

To test the relative dependence on these five major neutrophil-targeted chemoattractant receptors in protecting the brain from *C. albicans* infection, we infected mice deficient in CCR1, CXCR1, CXCR2, LTB4R1 or FPR1 and measured neutrophil recruitment and fungal brain burdens. We found no involvement of the CCL3/CCR1, CXCL5/CXCR1, LTB4/LTB4R1 or fMLP/FPR1 axes in controlling fungal infection of the brain, in line with the normal recruitment of neutrophils early in infection in *Ccr1<sup>-/-</sup>, Cxcr1<sup>-/-</sup>, Ltb4r1<sup>-/-</sup> and Fpr1<sup>-/-</sup> animals (Fig. 2c,d and Supplementary Fig. 4). In contrast, we found that CXCR2-deficient mice had a significant reduction in neutrophil accumulation and a corresponding significant increase in fungal growth in the brain (Fig. 2c,d). These data demonstrate the importance of the CXCR2 chemokine axis in neutrophil-mediated protection against *C. albicans* infection of the brain.

Next, we wondered which CXCR2 ligand may mediate the protective neutrophil recruitment to the fungal-infected brain. For that, we infected mice with a genetic deletion of the potent neutrophil chemoattractant Cxcl1/KC. Strikingly, these animals had decreased recruitment of neutrophils to the brain following infection and exhibited a similar CNS invasion susceptibility phenotype to the Cxcr2<sup>-/-</sup> mice (Fig. 2c,d). Therefore, we conclude that the CXCL1/CXCR2 chemokine axis is critical for protection against *C. albicans* brain invasion by promoting protective neutrophil recruitment. Importantly, this data indicates that the reduction in the accumulation of CXCL1 in the *C. albicans*-infected brain of Card9-deficient mice and in the human CARD9-deficient *C. albicans*-infected CSF is biologically relevant and significant<sup>13</sup>. 
Production of CXCL1 is Dependent on IL-1β in the Fungal-Infected Brain

Since we found that both IL-1β and CXCL1 were required for protection, we investigated whether their activation in the C. albicans-infected brain was sequential or parallel. For this, we measured production of IL-1β and CXCL1 in brain homogenates at 24 hours post-infection in animals lacking these inflammatory mediators using ELISA. We found no difference in production of IL-1β in CXCL1-deficient infected brains; however, we discovered a significant defect in the production of CXCL1 in the absence of IL-1β during brain invasion (Fig. 3a). To gain further insight into the IL-1β-dependent cellular sources of CXCL1 in the infected mouse brain, we infected WT and IL-1β-deficient mice and used intracellular flow cytometry. We found that CXCL1 and pro-IL-1β are produced by multiple myeloid phagocytes in the fungal-infected brain, including resident microglia, the most numerous immune cells in the brain, as well as recruited Ly6C^hi inflammatory monocytes which have been implicated in the control of C. albicans CNS invasion, and neutrophils themselves (Fig. 3b). Interestingly, Il1b^-/- microglia recovered from C albicans-infected brains had a profound defect in CXCL1 production, exhibiting significant reductions under every ex vivo restimulation condition tested (Fig. 3c). Ly6C^hi monocytes isolated from Il1b^-/- C. albicans-infected brains produced less CXCL1 when restimulated ex vivo with LPS, although we were unable to detect a difference under non-stimulated or zymosan-stimulated conditions, unlike microglia. In contrast, neutrophil production of CXCL1 did not differ significantly between the two groups of mice (Fig. 3c). Therefore, we conclude that IL-1β is required for subsequent CXCL1 production from resident microglia and recruited inflammatory monocytes, which in turn promotes the recruitment of CXCR2-expressing neutrophils to the fungal-infected brain (Fig. 2c).
Candidalysin Promotes IL-1β and CXCL1 Production and Protective Neutrophil Recruitment to the Fungal-Infected Brain

Mouse gene knockout experiments allowed us to map the complex host pathway promoting protection against C. albicans infection of the brain, in which IL-1β/IL-1R/MyD88 signaling activates production of CXCL1 via resident microglia and recruited inflammatory monocytes to mobilize neutrophils into the CNS early during infection. To identify the pathogen-associated factors that are required for induction of the IL-1β/CXCL1 protective host pathway in vivo, we infected animals with C. albicans strains lacking known virulence factors and assessed neutrophil recruitment and production of IL-1β and CXCL1 in the infected brain.

C. albicans hyphae are the predominant CNS-invasive morphological form of C. albicans and hyphal formation is associated with important virulence traits such as production of secreted toxins and proteases, adhesion, invasion, biofilm formation, and promotes immune system activation. Thus, we first asked whether neutrophil recruitment and control of C. albicans CNS invasion was impaired during infection with the hgc1ΔΔ C. albicans strain which is unable to filament in vivo. Indeed, infection with hypha-deficient hgc1ΔΔ C. albicans significantly impaired neutrophil recruitment and enhanced fungal CNS tissue invasion relative to the isogenic WT C. albicans strain (Fig. 4a). This data indicates that filamentation is not required for C. albicans invasion of brain tissue, in contrast to other organs such as the kidney, and that hyphal-specific fungal factors promote protective neutrophil recruitment into the C. albicans-infected brain.
Candidalysin is a recently-described peptide toxin encoded by the \textit{ECE1} gene and expressed exclusively by \textit{C. albicans} hyphae\textsuperscript{37, 38}. Candidalysin has been shown to mediate epithelial cell damage via pore formation in the plasma cell membrane resulting in IL-1\(\alpha\) release and pro-inflammatory cytokine production. As a result, Candidalysin-null mutants are highly attenuated in mouse models of oropharyngeal and vulvovaginal candidiasis\textsuperscript{37, 39, 40}. We hypothesized that Candidalysin activates the IL-1\(\beta\)/CXCL1 host pathway in the CNS. We found that the lack of Candidalysin promoted brain infection, and that this phenotype was specific to the Candidalysin peptide since mutant strains deficient in the entire gene (\textit{ece1}Δ/Δ) or specifically in the Candidalysin-encoding portion of the gene (\textit{ece1}Δ/Δ + \textit{ECE1}Δ\textsubscript{184-279}) were both hyper-virulent for brain invasion following \textit{C. albicans} challenge (Fig. 4b).

The increased ability of the Candidalysin-null mutants to proliferate within the brain directly correlated with the degree of neutrophil recruitment. We found a near absence of neutrophils in the brains of WT animals infected with Candidalysin-null strains and observed hyphal forms growing in the brain parenchyma with no surrounding neutrophilic reaction (Fig. 4c). In contrast, the Candidalysin-producing parental strain and the re-integrant control strain caused a significant induction of neutrophil recruitment at 24 hours post-infection, and these neutrophils clustered around invading fungal hyphae (Fig. 4c). In line with the absence of neutrophils in the brains of mice infected with Candidalysin-null strains, we found a significant reduction of both IL-1\(\beta\) and CXCL1 in brain homogenates from animals infected with these strains (Fig. 4d). This data shows that Candidalysin is a key fungal factor that activates the IL-1\(\beta\)/CXCL1 protective pathway \textit{in vivo}. Thus, in contrast to its role in the oral mucosa, Candidalysin acts as an avirulence factor in the brain by instigating protective host immunity in
the CNS, underscoring the tissue-specific opposing roles that a microbial factor may play during infection with the same pathogen\(^{41,42}\).

**Hyphal Secreted Aspartyl-Proteases Do Not Promote Neutrophil Recruitment to the Brain**

We next wondered whether other secreted proteins associated with *C. albicans* hyphae formation also activated protective neutrophil responses in the brain. Secreted aspartyl proteases (Saps) are a group of enzymes with extracellular proteolytic activity that are highly linked to virulence with several lines of evidence indicating that Saps are important for *C. albicans* pathogenicity\(^{43}\). Importantly, *C. albicans* Saps promote neutrophil recruitment in the context of vulvovaginal candidiasis in mice\(^ {44,45}\). Expression of the SAP4-6 subfamily is coordinately regulated with hyphal formation\(^ {43}\), therefore we asked whether these hyphal-associated Saps contributed towards virulence in the brain during invasive infection. We found that WT animals infected with the triple-deficient strain sap4/5/6\(\Delta/\Delta\) had comparable brain fungal burdens to animals infected with the complemented control strain (Fig. 4e). In line with this, we saw no difference in the neutrophil recruitment to the brains of these animals, indicating that *C. albicans* Saps exhibit tissue-specific roles in promoting neutrophil recruitment during infection\(^ {44,45}\) (Fig. 4e). Therefore, the induction of protective CNS neutrophil recruitment is activated by Candidalysin, and not by other *C. albicans* hyphae-secreted enzymes.

**Microglia are a Selective Myeloid Cellular Source of Candidalysin-Dependent Production of IL-1β and CXCL1**
Since we identified Candidalysin as a specific fungal factor controlling the activation of host immunity in the brain, we next sought to define the immune cells that were responding to Candidalysin during infection. For this, we infected WT mice with either the parental strain of \textit{C. albicans} (BWP17) or the Candidalysin-null strain (\textit{ece1Δ/Δ}), and then analyzed production of IL-1β and CXCL1 using intracellular flow cytometry at 24 hours post-infection (Fig. 5). In both cases, we found no production of IL-1β or CXCL1 by CD45<sup>-</sup>CD102<sup>+</sup>CD31<sup>+</sup> endothelial cells (data not shown).

Although all phagocytes in the brain produced both IL-1β and CXCL1 to recruit neutrophils, microglia were the only population to exhibit a dependence on Candidalysin, since microglia isolated from \textit{ece1Δ/Δ}-infected brains produced significantly less IL-1β \textit{ex vivo} (Fig. 5a). We observed an even stronger reduction in CXCL1-producing microglia from \textit{ece1Δ/Δ}-infected brains (Fig. 5b), in line with our earlier data which indicated a dependence on IL-1β for production of this chemokine (Fig. 3). In contrast, Ly6<sup>+</sup>C<sup>hi</sup> monocytes and neutrophils did not demonstrate a dependence on Candidalysin for IL-1β and CXCL1 production, suggesting that other as yet unidentified fungal factors are responsible for activating this pathway in these phagocytes. Taken together, our data show that Candidalysin acts on microglia to stimulate IL-1β release, which in turn drives CXCL1 production that is required for protective neutrophil recruitment to the brain.

\textbf{Candidalysin Acts Via p38-cFos Activation to Drive IL-1β Production from Microglia}

\textbf{Whereas Candidalysin-Induced CXCL1 Production from Microglia Requires Signals from Astrocytes Acting \textit{in trans}}
To gain mechanistic insights into the pathways controlling microglia responses to Candidalysin, we used the microglia cell line BV-2, a known model for primary murine microglia. We cultured BV-2 cells in the presence of synthetic Candidalysin and measured IL-1β and CXCL1 in the culture supernatants by ELISA. In line with our in vivo work, we found a time- and dose-dependent IL-1β response of the BV-2 cells in response to Candidalysin (Fig. 6a). We did not detect CXCL1 protein from BV-2 cells stimulated under these conditions. We first considered that this could be due to damage induced by Candidalysin that may have prevented the BV-2 cells from producing subsequent CXCL1, which according to our in vivo data, would occur downstream of IL-1β production (Fig. 3). Indeed, as previously shown for epithelial cells, Candidalysin mediated cell damage to BV-2 microglia in a dose-dependent manner (Fig. 6b). Alternatively, additional signals beyond IL-1β, derived from non-microglial CNS cells, might be required for the microglial CXCL1 induction in the brain, acting in trans. To test this hypothesis, we co-cultured BV-2 cells with immortalized C8-D1A astrocytes in the presence of Candidalysin and measured IL-1β and CXCL1 in the supernatants. We chose astrocytes since these cells have been previously shown to respond to IL-1β to produce inflammatory mediators, including CXCL1, in other models of CNS inflammation. These experiments revealed that astrocytes respond to Candidalysin to produce CXCL1 (Fig. 6c), but not IL-1β (data not shown), and that levels of CXCL1 significantly increase when the astrocytes and microglia were co-cultured together (Fig. 6c). To confirm that microglia are a relevant cellular source of the CXCL1 detected in these microglia-astrocyte supernatants, we performed intracellular staining for CXCL1 and found that BV-2 microglia are significant producers of CXCL1 in response to Candidalysin, but only when astrocytes were present (Fig. 6c).
Therefore, astrocytes provide additional signals to microglia that are needed to drive CXCL1 production in response to Candidalysin.

We next focused on understanding the pathway activated by Candidalysin in BV-2 microglia that is critical for IL-1β production. Candidalysin has been previously shown in epithelial cells to activate c-Fos in a p38-dependent manner, as well as the phosphatase MKP-1. We thus asked whether the same pathways are activated by Candidalysin in microglia. We stimulated BV-2 cells with Candidalysin, and measured induction of c-Fos and MKP-1 by western blot. We found that Candidalysin sequentially activated MKP-1 and c-Fos in a dose-dependent manner (Fig. 6e). Furthermore, chemical inhibition of p38 or c-Fos significantly reduced IL-1β release by Candidalysin-stimulated BV-2 cells (Fig. 6f). These data demonstrate that microglia produce IL-1β in response to Candidalysin using a pathway that involves activation of p38 and c-Fos.

**Activation of the Protective IL-1β-CXCL1 Response by Microglia Requires CARD9**

CARD9 deficiency is the only known risk factor that uniquely predisposes to CNS candidiasis in the absence of iatrogenic intervention, associated with a brain- and fungal-specific defect in neutrophil recruitment in the *C. albicans*-infected CNS (Fig. 1a). We first examined whether CARD9 deficiency causes developmental defects in resident microglia. We found that *Card9*-/- microglia exhibited no defects in abundance or activation markers at steady state and accumulated in similar numbers as WT microglia after *C. albicans* infection (Supplementary Fig. 5). Since *C. albicans* activates the IL-1β-CXCL1 axis in microglia to regulate protective neutrophil recruitment to the brain, we next aimed to analyze the dependence on CARD9 for
induction of this pathway in microglia after infection \textit{in vivo}. We hypothesized that CARD9 is required for these functions, as microglia highly express CARD9 and we previously found reduced transcription of CXC chemokines by these cells when FACS-sorted from the brain of \textit{C. albicans}-infected \textit{Card9}\textsuperscript{-/-} mice\textsuperscript{13}. To test this, we infected \textit{Card9}\textsuperscript{+/+} and \textit{Card9}\textsuperscript{-/-} animals with wild-type \textit{C. albicans}, isolated myeloid cells from the brain and measured pro-IL-1\(\beta\) and CXCL1 production by intracellular staining following \textit{ex vivo} restimulation, as before. Using this approach, we found a significant decrease in the frequency of CXCL1\(^+\) and pro-IL-1\(\beta\)\(^+\) cells in the fungal-infected \textit{Card9}\textsuperscript{-/-} brain, and that these decreases mapped to microglia which exhibited a dependence on CARD9 for both pro-IL-1\(\beta\) and CXCL1 production during \textit{C. albicans} brain invasion (Fig. 7\(\text{a,b}\)).

Since the production and secretion of mature IL-1\(\beta\) depends on expression of pro-IL-1\(\beta\) and the consecutive processing of pro-IL-1\(\beta\) into mature IL-1\(\beta\), we then asked whether microglia depend on CARD9 for IL-1\(\beta\) production via regulation of pro-IL-1\(\beta\) transcription and/or inflammasome activation. For this, we FACS-sorted microglia from WT and \textit{Card9}\textsuperscript{-/-} brains infected with the Candidalysin-expressing BWP17 \textit{C. albicans}, and examined \textit{Il1b} transcription by qRT-PCR as well as levels of pro-IL-1\(\beta\) and cleaved and pro-caspase-1 by western blot. These experiments revealed a significant decrease in \textit{Il1b} transcription in \textit{Card9}\textsuperscript{+/+} microglia during infection, which we confirmed at the protein level by western blot (Fig. 7\(\text{c,d}\)). These data are in line with the previously reported CARD9-dependent regulation of pro-IL-1\(\beta\) transcription in bone marrow-derived dendritic cells during viral infection\textsuperscript{50}. We also found a significant reduction in cleaved caspase-1 in \textit{Card9}\textsuperscript{-/-} microglia (Fig. 7\(\text{d}\)), indicating that Card9 operates at the levels of both pro-IL-1\(\beta\) transcriptional regulation and inflammasome activation.
for IL-1β production. Given that c-Fos mediates Candidalysin-induced IL-1β production by BV-2 cells (Fig. 6e,f), we measured c-Fos expression in FACS-sorted WT and Card9−/− microglia by western blot and found significantly decreased c-Fos expression in Card9−/− microglia (Fig. 7d).

Given the decreased caspase-1 cleavage in Card9−/− microglia (Fig. 7d), we next examined the levels of NLRP3 inflammasome in FACS-sorted WT and Card9−/− microglia by western blot. We focused on NLRP3 because CARD9 was reported to negatively regulate NLRP3 activation during macrophage Salmonella infection51, and we recently showed that Candidalysin activates NLRP3 in bone marrow-derived macrophages52. We found significantly decreased NLRP3 protein expression in Card9−/− microglia (Fig. 7e). Of interest, Nlrp3−/− animals had significantly decreased accumulation of neutrophils to the C. albicans-infected brain and increased fungal load after infection, in line with a potential contributory role for Card9-dependent NLRP3-inflammasome activation for protective neutrophil influx in the fungal-infected CNS (Fig. 7f). Taken together, these results demonstrate that microglia require CARD9 for c-Fos activation and for production of mature IL-1β via regulating transcription of Il1b and inflammasome activation, at least partly via NLRP3, in order to activate the IL-1β-CXCL1 axis in response to C. albicans invasion, which is needed for protective neutrophil recruitment to the C. albicans-infected brain.

Microglia-specific CARD9 Deficiency in Mice Impairs Neutrophil Recruitment and Causes Brain-Specific Susceptibility to Fungal Invasion
Given the impaired production of IL-1β and CXCL1 by Card9−/− microglia in vivo (Fig. 7), we next aimed to directly examine the impact of genetic deletion of Card9 specifically within the microglia compartment on CNS neutrophil recruitment and on the control of fungal brain invasion. For this, we utilized mice expressing tamoxifen-inducible Cre recombinase under the Cx3CR1 promoter (Cx3cr1CreER)53, which has been extensively used to genetically manipulate long-lived Cx3CR1+ microglia while leaving short-lived Cx3CR1+ monocytes and monocyte-derived macrophages unaffected54, 55. We bred Cx3cr1CreER animals to Card9-floxed mice29, tamoxifen-pulsed the progeny soon after weaning to activate Cre expression and then waited 4-6 weeks to allow replenishment of short-lived non-microglia Cx3CR1+ cells from the bone marrow, while long-lived microglia cells remain Card9-deficient (Supplementary Fig. S6). Infection of Card9fl/flCx3CR1CreER+/− animals with C. albicans revealed a significant dependence on Card9 expression by the long-lived CX3CR1+ cellular compartment for control of fungal growth within the brain (Fig. 8a). Importantly, control of fungal infection in an unrelated organ, the kidney, was unaffected in microglia-specific conditional Card9−/− mice (Fig. 8a).

To analyze whether the increased susceptibility to brain infection that we observed in Card9fl/flCx3CR1CreER+/− mice was related to defects in neutrophil recruitment, we quantified neutrophils that had accumulated within the brains of Card9fl/flCx3CR1CreER+/− mice and their Cre-negative littermates using flow cytometry. Importantly, we found that deletion of Card9 specifically within the microglia compartment significantly reduced the protective early influx of neutrophils into the brain following fungal infection (Fig. 8b), which correlated with significant decreases in the expression of microglial pro-IL1β and CXCL1 in the conditional Card9−/− mice (Fig. 8c). Together, our data shows that CARD9-expressing microglia are required for control
of fungal brain invasion, in part by responding to Candidalysin secreted by the invading fungus, to produce IL-1β-induced CXCL1, which recruits CXCR2-expressing neutrophils that are required for CNS fungal clearance (Fig. 8d).
Discussion

Herein, we demonstrate the critical contribution of IL-1β and CXCL1 in promoting protective neutrophil recruitment to the fungal-infected CNS in vivo. We identify microglia as major early producers of IL-1β and CXCL1 during C. albicans CNS invasion and show that the secreted fungal toxin Candidalysin is a critical pathogen-derived factor driving microglial production of neutrophil-recruiting IL-1β and CXCL1, via activation of p38-cFos signaling. Importantly, we show that microglial production of IL-1β and CXCL1 relies on CARD9 in the C. albicans-infected CNS and we demonstrate that specific deletion of CARD9 in microglia impairs neutrophil recruitment and control of fungal CNS invasion. Taken together, our study offers novel insights into the network of host and fungal factors that protect the CNS against fungal invasion and unveil the mechanism by which inherited CARD9-deficiency promotes susceptibility to CNS fungal disease.

Systemic candidiasis is a leading cause of nosocomial bloodstream infection in the US with >40,000 affected patients per year and mortality that exceeds 50% despite antifungal therapy. Neutropenia is the major risk factor for developing systemic candidiasis and Candida invasion of the CNS in particular. In addition, CNS candidiasis is a particularly prevalent complication of systemic candidiasis in low-birth weight neonates and it also occurs as an iatrogenic complication following neurosurgical procedures. Strikingly, biallelic mutations in the CLR/Syk signaling adaptor CARD9 result in a primary immunodeficiency disorder (PID) characterized by heightened selective susceptibility to fungal infections of which CNS candidiasis is a hallmark. In fact, CARD9 deficiency is the only known PID that
results in fungal-specific infection susceptibility without predisposition to other infectious or non-infectious manifestations, and the only PID that predisposes to fungal disease in which CNS is the primary target organ\textsuperscript{35}. We previously demonstrated that this predilection for developing Candida CNS infections in the context of CARD9-deficiency is caused by a fungal- and brain-specific defect in neutrophil recruitment to the site of CNS invasion\textsuperscript{13}. CNS neutropenia has now been confirmed in other CARD9-deficient patients with CNS candidiasis who carry different CARD9 mutations\textsuperscript{24, 59, 60}. Nonetheless, how CARD9 mediates protective neutrophil trafficking into the C. albicans-infected CNS has remained unclear. Thus, in the current study, we systematically investigated the cellular and molecular factors that promote protective neutrophil recruitment to the CNS during fungal invasion and their dependence on functional CARD9 for induction \textit{in vivo} with a goal to decipher the pathogenesis of inherited CARD9-deficiency.

We first examined the individual contribution of the CARD9-coupled CLRs Dectin-1, Dectin-2, Dectin-3 and Mincle in mediating neutrophil recruitment and fungal growth control in the C. albicans-infected CNS. Our findings demonstrate significant functional redundancy among these CLRs, as only genetic deficiency of all four known CARD9-coupled CLRs approached the defective neutrophil trafficking and impaired control of fungal proliferation in the CNS seen with CARD9-deficiency, which may indicate that there may as of yet undiscovered CARD9-coupled receptors operating during fungal infection to provide tissue-specific protection. Lack of Dectin-1 or Dectin-2 alone did result in a modest impairment in restricting CNS fungal invasion without an accompanying decrease in neutrophil recruitment but with a decrease in fungal uptake, suggesting that Dectin-1 and Dectin-2 may regulate the effector function of
neutrophils and/or other recruited and/or resident phagocyte populations in the fungal-infected CNS. Of note, deficiency in the CARD9 partner MALT1 led to a significant increase in CNS fungal invasion, which was independent of impaired neutrophil recruitment to the infected tissue. This finding underscores the differential mechanistic roles of the distinct components of the CBM signalosome in promoting antifungal immunity and implies that MALT1−/− patients may exhibit enhanced susceptibility to developing CNS fungal disease. Hence, future studies will be required to examine the mechanisms by which Malt1 promotes effective host defense against C. albicans in the CNS and to determine whether and by which mechanisms other Card9-partners such as Bcl10 or Trim62 may promote anti-Candida immunity in the CNS.

Our analysis of several mouse lines deficient in cytokine and chemokine circuits uncovered the critical contribution of an IL-1β/CXCL1-mediated protective host pathway for driving neutrophil recruitment and control of fungal invasion in the C. albicans-infected CNS. Indeed, animals lacking IL-1β and its receptor IL-1R or adaptor protein MyD88, and mice lacking CXCL1 and its receptor CXCR2 are highly susceptible to CNS candidiasis caused by impaired neutrophil accumulation to the infected brain. CXCR2 is an important mediator of neutrophil trafficking and promotes neutrophil accumulation during viral infection, parasitic meningitis and fungal pneumonia. Here, we identify CXCR2 as the chemokine receptor that mediates neutrophil accumulation to the C. albicans-infected brain, via binding CXCL1. In contrast, we found no role for CCR1, CXCR1 or LTB4R1, although we and others have previously found that these receptors regulate neutrophil recruitment and function to the C. albicans-infected kidney and lung. These studies further demonstrate the organ-specific and context-specific...
dependence on chemokine receptors and their ligands for the induction of protective host immunity.

Upstream of the neutrophil-recruiting CXCL1/CXCR2 axis, we show that IL-1β is required for efficient CXCL1 induction, since animals deficient in IL-1β produce significantly less CXCL1 in the *C. albicans*-infected brain. This data is in line with earlier work which showed that IL-1β-induced production of CXCL1 is required for neutrophil accumulation to the bacterial-infected peritoneum and during autoimmune, traumatic or bacterial-induced neuroinflammation. Importantly, microglia, the resident phagocytes of the CNS, which are the first to encounter invading fungi and by far the most numerous phagocytes in the *C. albicans*-infected brain, are the primary cellular source of IL-1β-dependent CXCL1 production in vivo. During mucosal candidiasis, the IL-1R is also required for neutrophil accumulation to the oral mucosa, as we have shown for systemic infection in the brain. However, further attesting to the presence of tissue-specific anti-*Candida* immune response cues, the IL-1R-dependent response in the oral epithelium is largely controlled by IL-1α released by damaged keratinocytes, whereas we found that IL-1α plays a modest role in the control of brain fungal invasion. In fact, IL-1α release by epithelial cells, in both the oral and vaginal mucosal barriers, is driven by exposure to the fungal secreted toxin Candidalysin. Candidalysin is secreted by fungal hyphae and enables the establishment of mucosal infections by *C. albicans*, since strains deficient in Candidalysin are avirulent in these models. In contrast to these findings, we found that Candidalysin does not promote virulence of *C. albicans* in the brain. Instead, strains unable to produce Candidalysin are hyper-virulent for this
tissue, associated with decreased IL-1β and CXCL1 production in the C. albicans-infected CNS and a resultant disruption in the recruitment of protective neutrophils. These results indicate that Candidalysin is not only a classical virulence factor, but also an immune modulator, which can exert specific effects on the immune system that are context-dependent. Indeed, C. albicans exhibits markedly variable transcriptional and morphological responses within different organs, which drive distinct virulence programs that can profoundly influence the resulting immune response. We propose that this dual function of Candidalysin is the result of a co-evolutionary event; the fungus has developed an efficient peptide toxin to damage host membranes, and, in response, the host has evolved a sensitive Candidalysin detection system to defend itself against this common mucosal pathogen. Whether Candidalysin is recognized by a specific innate receptor on microglia to mediate the protective IL-1β-CXCL1 axis is unclear, since this toxin mediated significant cellular damage which could also be activating brain-resident glial cells. Therefore, identifying the potential receptor(s) that recognize(s) Candidalysin in host epithelial and immune cells is an important direction of future research.

Our findings indicate that Candidalysin is a selective fungal hyphal-associated factor that activates the protective neutrophil response in the C. albicans-infected brain, and that Candidalysin-dependent production of IL-1β and CXCL1 in vivo selectively localizes to microglial cells within the myeloid compartment. Microglia are a self-renewing macrophage population that contribute towards neuroinflammation in neurodegenerative disorders and promote clearance of pathogens and dead cells from the CNS. Yet, mechanistic insights into how microglia elicit protective antifungal immunity are lacking. Here, using an in vitro
model of BV-2 microglial cells, we demonstrate that Candidalysin induces IL-1β production via
activation of p38 and c-Fos signaling in microglia. Downstream of IL-1β secretion by microglia,
we show that additional signals deriving from astrocytes acting \textit{in trans} are required for
microglia to secrete CXCL1. Whether direct microglia-astrocyte contact is required or an
astrocyte-derived soluble factor acting on microglia is needed remains unknown. Indeed,
miroglia have been shown to interact with astrocytes and/or recruited leukocytes in other
settings to drive or suppress inflammation\textsuperscript{72, 73, 74}. Therefore, the molecular factors that underlie
the cross-talk occurring between microglia and astrocytes within the fungal-infected brain
warrant further investigation.

After delineating the microglia-mediated, IL-1β/CXCL1-dependent pathway that is activated \textit{in}
vivo in the \textit{C. albicans}-infected brain to promote protective neutrophil recruitment, we
examined the dependence of this pathway on functional CARD9. We show that CARD9 is
critical for the production of IL-1β and CXCL1 by microglia \textit{in vivo} in the infected CNS. In
keeping with this, CXCL1 was decreased in the \textit{Card9}\textsuperscript{-/-} \textit{C. albicans}-infected mouse brain and
in the \textit{CARD9}\textsuperscript{-/-} \textit{C. albicans}-infected human CSF\textsuperscript{13}. We used qRT-PCR and western blot
analyses in primary microglia FACS-sorted from WT and \textit{Card9}\textsuperscript{-/-} mice to show that CARD9
regulates c-Fos activation and operates at the levels of both transcriptional pro-IL-1β regulation
and inflammasome activation for IL-1β generation, with NLRP3 being at least partly involved,
as previously shown in response to \textit{Microsporum} infection\textsuperscript{75}.

To directly evaluate the role of microglia-expressed Card9 in anti-\textit{Candida} host defense \textit{in}
vivo, we generated conditional Card9 knockout mice that lack Card9 in microglia, infected
them with \textit{C. albicans}, and evaluated their neutrophil-recruiting capacity and ability to suppress fungal invasion in the \textit{C. albicans}-infected CNS. Importantly, we found that specific deletion of microglial Card9 results in decreased microglial IL-1\(\beta\) and CXCL1 production, defective neutrophil recruitment and impaired control of fungal proliferation in the infected CNS. Taken together, these data shed light into the pathogenesis of inherited CARD9-deficiency by outlining a pathway of CARD9-dependent microglial production of sequential IL-1\(\beta\) and CXCL1 that promotes protective neutrophil recruitment into the fungal-infected CNS (Fig. 8d).

Several questions arise from our data that require further investigation. It will be important to examine the role of Card9 in promoting other innate functions of microglia beyond mediating protective neutrophil recruitment that may contribute towards \textit{C. albicans} uptake, killing and containment within the CNS. Furthermore, it will be important to use corresponding Card9 conditional knockout mouse lines to determine the potential contribution of astrocyte-, monocyte- and neutrophil-intrinsic Card9 in mediating protective neutrophil recruitment, phagocyte effector function, and antifungal host defense in the CNS. Indeed, the phenotype of the microglia-specific conditional knockout mice is less severe than that of Card9\(-/-\) mice, which may reflect the important role of astrocytes (which express Card9 following \textit{Candida} infection\textsuperscript{13}) in priming CXCL1 production by microglia. The latter observation could also relate to potential experiment-to-experiment variation in recombination efficiency post-oral tamoxifen administration and/or a potential requirement of instituting Card9 deficiency in microglia \textit{in utero} compared to the 5-6 week-age that is feasible with the available CreER system. In addition, future work should examine the tissue-specific dependence on Card9 for promoting production of sequential IL-1\(\beta\) and CXCL1 for neutrophil recruitment by non-CNS tissue-
resident macrophages. It is possible that Card9 is differentially utilized downstream of CLR
engagement by different tissue-resident macrophages such as splenic macrophages or
Kupffer cells for the production of IL-1β and CXCL1 and neutrophil recruitment, as previously
shown for macrophages and dendritic cells\textsuperscript{76}. Therefore, the IL-1β/CXCL1 pathway we
identified in the CNS may not rely on functional Card9 for induction in non-CNS tissue-resident
macrophages. Alternatively, lack of Card9-dependent sequential production of IL-1β and
CXCL1 in non-CNS tissues may be compensated by other pathways that are operational in
these tissues, but not in the CNS. Beyond understanding the pathogenesis of inherited
CARD9-deficiency, our findings have important implications for recognizing the potential fungal
infection risk in patients who are increasingly receiving inhibitors that target Syk, which lies
upstream of CARD9, for the treatment of autoimmune conditions, graft-versus-host disease,
and hematological malignant diseases\textsuperscript{77, 78, 79}. Thus, surveillance of such patients and future
research in conditional Syk knockout mice will be required to determine the risk of CNS fungal
disease in these patients.

In summary, we present evidence of an intricate host immune pathway that mediates
protection of the CNS from invading fungi and is selectively activated in microglia by the
secreted peptide toxin Candidalysin. Invading fungi drive IL-1β and CXCL1 production from
CARD9-expressing microglia in a sequential manner, which promotes the recruitment of
CXCR2-expressing neutrophils to the brain that are needed to control CNS infection. This work
uncovers the complex interactions occurring between the host and the most common human
fungal pathogen in the CNS, and sheds novel mechanistic light into the pathogenesis of
inherited CARD9-deficiency.
Materials and Methods

Mice

Animals (males and females) were used at 8-12 weeks of age and were maintained in individually-ventilated cages under specific pathogen-free conditions at the 14BS facility at the National Institutes of Health (Bethesda, MD, USA), the Memorial Sloan Kettering Cancer Center Comparative Medicine Shared Resources (New York, NY, USA), or the Medical Research Facility at the University of Aberdeen (UK). The following strains (and their respective WT controls/littermates) were obtained from the NIAID Taconic contract; Cxcr2−/−, Il1r−/−, Ltb4r1−/−, Fpr1−/−. All other strains and their respective controls/littermates were bred in-house at the NIH (Clec7a−/−, Clec4n−/−, Clec4e−/−, Myd88−/−, Ccr1−/−, Cxcr1−/−, Cxcl1−/−, Il1a−/−, Il1b−/−, Il1a−/−Il1b−/−, Nlrp3−/−, Card9fl/flCx3CR1CreER+/−), Memorial Sloan-Kettering Cancer Center (Clec7a−/−Fcer1g−/−), University of Aberdeen (Clec4d−/−), or USUHS (Malt1−/−). Mice homozygous for the Card9tm1a allele were purchased from the Wellcome Trust Sanger Institute (EUCOMM Project No. 44813), and these animals were bred with the FLPer deleter strain (Jackson Laboratories) to remove the FRT-flanked knock-out first cassette, generating Card9tm1tc homozygous mice (referred to as Card9fl/fl in this manuscript). Homozygous Card9fl/fl animals were bred with heterozygous Cx3cr1CreER transgenic animals (Jackson Laboratories) to generate Card9fl/flCx3cr1CreER+/− mice and littermate controls. Soon after weaning (~5-6 weeks old), Card9fl/flCx3cr1CreER+/− mice and their controls were treated with two 10mg doses of tamoxifen (Sigma) administered in corn oil by oral gavage, given 48 hours apart. After 4-6 weeks, these animals were infected and analyzed as outlined in the Figure legends. All experimentation
conformed to conditions approved by the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases.

**Candidiasis Model and Fungal Burden Determination**

*Candida albicans* strains used in this study were SC5314, BWP17, *ece1ΔΔ*, *ece1ΔΔ+ECE1*,

*ece1ΔΔ+ECE1Δ184-279* 37, CAI4+Clp10 and *sap4/5/6ΔΔ*, and *hgc1ΔΔ* and *hgc1ΔΔ + HGC1*36.

Yeast was serially passaged 3 times in YPD broth, grown at 30°C with shaking for 18-24 hours at each passage. Yeast cells were washed in PBS, counted, and injected intravenously via the lateral tail vein. Animals were infected with 1.3x10^5 CFU for analysis at 24 hours post-infection, or 7x10^4 CFU for analysis at 72 hours post-infection, unless otherwise stated in the corresponding Figure legends. For analysis of brain fungal burdens, animals were euthanized and brains weighed, homogenized in PBS, and serially diluted before plating onto YPD agar supplemented with Penicillin/Streptomycin (Invitrogen). Colonies were counted after incubation at 37°C for 24-48 hours.

**Analysis of Brain Neutrophil Recruitment by FACS**

Leukocytes were isolated from brains using previously described methods68, resuspended in PBS and stained with Live/Dead fluorescent dye (Invitrogen) for 10 minutes on ice. Cells were then stained with fluorophore-conjugated antibodies in the presence of anti-CD16/32 and 0.5% BSA for 30 minutes on ice. Samples were washed in PBS/0.5% BSA/0.01% sodium azide and acquired using the BD Fortessa instrument equipped with BD FACS Diva software (BD Biosciences). FlowJo (TreeStar) was used for the final analysis. Anti-mouse antibodies used in
this study were: CD45 (30-F11), CD11b (M1/70), both from eBiosciences, and Ly6G (1A8), Ly6C (AL-21), both from BD Biosciences.

**Histology**

Brains were removed from infected mice at the indicated time points and fixed in 10% formalin for 24 hours before embedding in paraffin wax. Tissue sections were stained with periodic acid-Schiff (PAS).

**Measurement of Cytokines and Chemokines in Brain Homogenates**

Infected brains were isolated at 24 hours post-infection and homogenized in 1mL PBS supplemented with 0.05% Tween20 and protease inhibitor cocktail (Roche). Homogenized brains were centrifuged twice to remove debris and resulting supernatants snap-frozen on dry ice and stored at -80 °C prior to analysis. IL-1β and CXCL1 concentrations in the homogenates was determined by ELISA (R&D Systems), following the manufacturers’ instructions.

**Ex Vivo Restimulations and Intracellular FACS Analysis**

Animals were infected with 2x10^5 CFU of the indicated C. albicans strains intravenously, and brain leukocytes isolated 24 hours later. For these experiments, brains were first digested in RPMI supplemented with 0.8 mg/mL Dispase (Gibco), 0.2mg/mL Collagenase Type 4 (Worthington), and 0.1 mg/mL DNAse (Roche) at 37°C for 30 minutes, then pipetted vigorously to create a homogenous suspension. These suspensions were centrifuged (1500 rpm, 5 minutes, 4°C), pellets resuspended in 5 mL 40% Percoll (GE Healthcare) and centrifuged again at 1700 rpm for 20 minutes at 4°C to remove myelin. Cell pellets were washed in RPMI
supplemented with 10% heat-inactivated foetal bovine serum and Penicillin/Streptomycin (Invitrogen), and added to FACS tubes for stimulations. Cells were incubated for 4 hours at 37°C in the presence of 5 µg/mL Brefeldin A (Sigma) and, where indicated, 1 µg/mL LPS (Sigma) or 62.5 µg/mL depleted zymosan (Sigma). After stimulation, cells were washed in PBS and stained for surface markers as above. Fixation/permeabilization was performed with the eBioscience Foxp3 staining kit, and staining for CXCL1 (IC4532R, from R&D Systems) or pro-IL-1β (NJTEN3; from eBioscience) performed overnight at 4°C. Samples were washed once in PBS/0.5% BSA/0.01% sodium azide prior to acquisition using the BD Fortessa instrument equipped with BD FACS Diva software (BD Biosciences). FlowJo (TreeStar) was used for the final analysis. CXCL1/pro-IL-1β positive cells were determined by employing similar staining and gating in animals deficient in these mediators (Cxcl1−/−, Il1b−/−) as negative controls.

**Cell Culture and Candidalysin Stimulation**

BV-2 cells were kindly provided by Dr Fulton Crews (UNC School of Medicine). BV-2 were maintained at 37°C, 5% CO₂ in RPMI supplemented with L-glutamine and HEPES (Corning), 10% heat-inactivated foetal bovine serum and Penicillin/Streptomycin (Invitrogen). DMEM media was used as the base media for C8-D1A culture, with the same supplements as listed above and cultured as for BV-2. For BV-2 single culture experiments, cells were lifted using cell scrapers and seeded into 24 well-plates at 5x10⁵ cells/well (BV-2) and left to adhere for 2 hours at 37°C with either: 50 ng/mL LPS (Sigma), T-5224 (APExBIO) and/or SB203580 (Adipogen); see Figure legends for details of each experiment. After 2 hours, recombinant Candidalysin peptide (Peptide Protein Research, UK) was added to the cells at the indicated concentrations and
For co-culture experiments, 3x10^5 C8-D1A were added to each well of a 24 well-plate and incubated overnight at 37°C. BV-2 cells and Candidalysin were added as described above. In both types of experiments, supernatants or cells were collected at the indicated time points after Candidalysin addition and analyzed for IL-1β and CXCL1 by ELISA (R&D Systems), CXCL1 staining by intracellular flow cytometry (see above), or by Western blot (see below).

**Western Blot Analysis**

Whole cell lysates were suspended in RIPA buffer containing protease and phosphatase inhibitors (Thermo Scientific). Lysates were separated in SDS-PAGE and transferred to a nitrocellulose membrane, 0.2 µm (Bio-Rad Laboratories). The membrane was incubated with the following primary antibodies: phospho-MPK1/MPK2 polyclonal [Ser296, Ser318] (Thermo Scientific) and c-Fos (Cell Signaling), IL-1β [3A6] (Cell Signaling), Caspase-1 p20 [Casper-1] (Adipogen Life Sciences) (Thermo Scientific), NLRP3 [D4D8T] (Cell Signaling). Normalization was performed by probing the membrane with β-Actin antibody (Cell Signaling).

Chemiluminescence detection was performed with Clarity™ Western ECL Substrate (Bio-Rad Laboratories), using the ChemiDoc™ MP Imaging System (Bio-Rad).

**FACS/MACS Sorting of Microglia**

WT animals were infected with 1.3 x 10^5 CFU SC5314 and euthanized at 24 hours post-infection. Brains were digested as above and leukocytes stained with sterile antibodies. Ly6C^hi monocytes (CD45^hi CD11b^+ Ly6C^hi Ly6G^-) and microglia (CD45^lo CD11b^+ Ly6G^- Ly6C^-) were FACS-sorted into sterile sorting buffer (HBSS supplemented with 2 mM EDTA, 10 % FCS, 100
U/mL penicillin, 100 μg/mL streptomycin) using a FACS Aria instrument for downstream qRT-PCR and western blot analyses. Purity of cells were greater than >95%, on average. In some experiments (qRT-PCR of CLRs in brain-resident microglia; Supplementary Fig. 1), microglia were instead sorted by magnetic separation using anti-CD11b microbeads (Miltenyi). Cells were then centrifuged (1500 rpm 5 minutes, 4 °C) and resuspended in Trizol for RNA purification or RIPA buffer for downstream western blot analysis. Depending on the experiment, up to 5 animals were pooled for individual sorts, or individual mice were analyzed separately (see Figure legends for details).

**Generation of cDNA and qRT-PCR**

RNA was extracted from sorted brain myeloid cells (defined using the gating strategy shown in Supplementary Fig. 2) using Trizol (Invitrogen) and the RNeasy kit (Qiagen) per the manufacturer’s protocol. Purified RNA was used as a template for cDNA generation using the qScript cDNA SuperMix kit (Quanta Biosciences) with oligo(dT) and random primers.

Quantitative PCR was performed by TaqMan detection (PerfeCTa qPCR FastMix ROX; Quanta BioSciences) with the 7900HT Fast Real-Time PCR System (Applied Biosystems). All qPCR assays were performed in duplicate and the relative gene expression of each gene was determined after normalization with GAPDH transcript levels using the \( \Delta\Delta CT \) method. TaqMan primers/probes (Clec7a, Clec4n, Clec4d, Clec4e, Il1b, Card9, Gapdh) were predesigned by Applied Biosystems.

**Statistics**
Statistical analyses were performed using GraphPad Prism 7.0 software. Details of individual tests are included in the figure legends. In general, data was tested for normal distribution by Kolmogorov-Smirnov normality test and analyzed accordingly by unpaired t-tests or Mann Whitney U-test. In cases where multiple data sets were analyzed, two-way ANOVA was used with Bonferroni correction. In all cases, $P$ values <0.05 were considered significant.
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References


**Figure Legends**

**Fig. 1:** CARD9-coupled C-type lectin receptors functionally compensate for one another for protective neutrophil recruitment to the fungal-infected brain. 

*a,* *Card9*<sup>−/−</sup> mice and their wild-type controls (n=4) were intravenously infected with *C. albicans* SC5314 and analyzed for neutrophil counts by flow cytometry at 24 hours post-infection (left; dose: 1.3x10<sup>5</sup> CFU) and fungal growth within the brain at 72 hours post-infection (right; dose: 7x10<sup>4</sup> CFU). 

*b,* Animals of the indicated genotype (n=6-12) were intravenously infected with *C. albicans* SC5314 (2x10<sup>5</sup> CFU for *Clec4d*<sup>−/−</sup> and *Clec7a*<sup>−/−</sup>*Fcer1g*<sup>−/−</sup> and their controls; 1.3x10<sup>5</sup> all others) and analyzed for neutrophil counts by flow cytometry at 24 hours post-infection and 

*c,* fungal burdens in the brain at 24 and 72 hours post-infection. 

*d,* *Malt1*<sup>−/−</sup> mice and their littermate controls (n=5-9) were infected as above and analyzed for fungal burdens in the brain (right) and neutrophil recruitment to the brain at 24 hours post-infection (left). In all cases, ‘wild type’ refers to appropriate matched control animals for each knock-out line for gender, age and genetic background. Individual points represent different mice. Data is pooled from 2 independent experiments and is shown as mean +/- SEM, and analyzed by unpaired two-tailed t-test or Mann Whitney U-test. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001.

**Fig. 2:** IL-1β and CXCL1 are critical for protective neutrophil recruitment to the fungal-infected brain. 

*a,b,* Animals deficient in elements of the IL-1R signaling pathway or 

*c,d,* chemokine receptors and their ligands, were infected (n=6-15) and analyzed for neutrophil recruitment at 24 hours post-infection (a,c) and control of fungal brain infection (b,d) as in Fig. 1. ‘Wild type’ refers to appropriate matched control animals for each knock-out line for gender,
age and genetic background. Individual points represent different mice. Data is pooled from 2-3 independent experiments and shown as mean +/- SEM, analyzed by unpaired two-tailed t-test or Mann Whitney U-test. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001.

**Fig. 3**: Production of CXCL1 is dependent on IL-1β in the fungal-infected brain. a, Wild type, Cxcl1−/− and Il1b−/− animals were infected as in Fig. 1 (n=5-7) and brains isolated at 24 hours post-infection and analyzed for CXCL1 or IL-1β production by ELISA. b, The relative proportions of myeloid cell populations (gated within live CD45+ singlets) in the uninfected and 24h infected WT brain (left), and the relative proportion of myeloid cell populations producing CXCL1 or pro-IL-1β in the 24h infected brain (right). For the latter, total CD45+CXCL1(or IL-1β)+ cells were first gated and then cell types defined within this initial gate using lineage markers (see below), using samples from the unstimulated condition. c, Wild type and Il1b−/− mice (n=3-4) were infected with 2x10^5 C. albicans and brain cells analyzed for CXCL1 production by intracellular flow cytometry 24 hours later. Brain cells were restimulated ex vivo with 62.5µg/mL depleted zymosan or 1µg/mL LPS for 4 hours in the presence of 5µg/mL Brefeldin A. Representative plots from the LPS-stimulated condition are gated on microglia (top; CD45^{int}Ly6G^−CD11b^+), Ly6C^hi monocytes (middle; CD45^{hi}Ly6C^{hi}Ly6G^−CD11b^+) and neutrophils (bottom; CD45^{hi}Ly6C^{int}Ly6G^{hi}CD11b^+), showing corresponding Cxcl1−/− cells as gating controls. ‘Wild type’ refers to appropriate matched control animals for each knock-out line for gender, age and genetic background. Individual points represent different mice. Data is pooled from 2 independent experiments and shown as mean +/- SEM, and analyzed by unpaired two-tailed t-test or Mann Whitney U-test. *P<0.05, **P<0.01.
**Fig. 4:** Candidalysin is a specific hyphal-secreted factor promoting neutrophil recruitment and control of fungal growth in the brain. a,b,c,e, Animals (n=6-13) were infected with 2x10^5 CFU of the indicated *C. albicans* strains (parental strains, closed symbols; deficient mutants, open symbols) and analyzed as in Fig. 1 for fungal burdens and neutrophil recruitment. Histology shown in (c) is from 24 hours post-infection, stained with PAS. Scale bar is 50µm. d, Whole brain homogenates from animals infected with indicated strains were isolated at 24 hours post-infection and analyzed for IL-1β and CXCL1 using ELISA. Individual points represent different mice. Data is pooled from 2-4 independent experiments and shown as mean +/- SEM, analyzed by unpaired two-tailed t-test or Mann Whitney U-test. *P<0.05, **P<0.01, ***P<0.005; ns = not significant.

**Fig. 5:** Microglia produce IL-1β and CXCL1 in a Candidalysin-dependent manner.
Animals (n=9-12) were infected with wild-type *C. albicans* (BWP17; closed circles/bars) or a Candidalysin-null strain (*ece1Δ/Δ*; open circles/bars), and brain cells isolated 24 hours later. Brain leukocytes were restimulated as in Figure 3, and intracellular staining for a, IL-1β and b, CXCL1 was analyzed by flow cytometry. Box-and-whisker plots show pooled data from 2-4 independent experiments. Representative staining is shown for LPS-stimulated microglia (gated as in Figure 3) from wild type mice infected with indicated strains, or BWP17-infected cytokine-deficient mutants as control. Data analyzed by unpaired two-tailed t-tests. *P<0.05, **P<0.01.

**Fig. 6:** Candidalysin activates IL-1β production from microglia via p38-cFos signaling and promotes CXCL1 production from microglia through interactions with astrocytes.
BV2 microglia were seeded into 24-well plates at 5x10^5 per well and left to adhere for 2 hours in the presence of 50 ng/mL LPS (for priming) before the addition of purified Candidalysin at the indicated concentrations. Cell culture supernatants were analyzed for a, IL-1β production or b, LDH release after 24 hours of stimulation. c-d, In some experiments, BV2 cells were co-cultured with 3x10^5 C8-D1A astrocytes, and CXCL1 production analyzed in the supernatant by ELISA or by intracellular flow cytometry. e, To measure cFos and pMKP1/2 activation, BV2 cells were stimulated with the indicated Candidalysin concentrations for 30 or 120 minutes and BV2 cells then lysed and analyzed for cFos and pMKP1/2 by western blot, normalizing to β-actin. f, BV2 cells were cultured in the presence of the indicated cFos and p38 inhibitors for 2 hours prior to stimulating with 20µM Candidalysin, and IL-1β measured in the supernatant by ELISA after 24 hours. Data is pooled from 2 independent experiments and is shown as mean +/- SEM, where each point represents an independent cell culture well (n=4-6). Data analyzed by one-way ANOVA. *P<0.05, **P<0.01, ***P<0.005, ****P<0.0001.

**Fig. 7:** CARD9 is required for the production of IL-1β, via regulation of pro-IL-1β transcription and inflammasome activation, and of CXCL1 in the fungal-infected brain. a,b, Card9^{+/+} and Card9^{-/-} animals (n=10-13) were infected with 2x10^5 CFU wild-type C. albicans (BWP17), and brain cells isolated 24 hours later. Brain leukocytes were restimulated as in Figure 4, and intracellular staining for pro-IL-1β and CXCL1 analyzed by flow cytometry in total CD45^{+} cells (LPS-stimulated condition shown) (a) or microglia alone (b). Box-and-whisker plots show pooled data from 4 independent experiments. Statistics shown compare pooled data and are normalized to Card9^{+/+} results. c, Microglia were FACS-sorted from pooled Card9^{+/+} and Card9^{-/-} animals at 24h post-infection and analyzed for Il1b expression by qRT-
PCR, or d,e, the indicated proteins by western blot. Graphs in (d,e) represent the band pixel density normalized to the wild type control, and are shown with mean +/- SEM and analyzed by unpaired two-tailed student t-tests. Example blots are representative of 3 independent FACS sorts/experiments; pooled data is shown in the graphs above. f, Nlrp3−/− animals and their wild-type controls were infected with 1.3x10^5 CFU C. albicans and analyzed for neutrophil recruitment to the brain 24h later (left) and fungal brain burdens at 72h post-infection (right), as described in Figure 1. *P<0.05, **P<0.01, ***P<0.005.

**Fig. 8:** CARD9 is required specifically in microglia for neutrophil recruitment and control of fungal invasion in the CNS. a, Card9fl/flCx3cr1CreER−/− and Card9fl/flCx3cr1CreER+/− littermates (n=8-13) were tamoxifen-pulsed at 4-5 weeks of age, left to rest for 4-6 weeks and then infected with 1.3x10^5 CFU C. albicans (SC5314) intravenously and analyzed for brain and kidney fungal burdens, b, neutrophil recruitment to the brain at 24 hours post-infection, and c, intracellular staining for pro-IL-1β and CXCL1, as described in Figure 3. Data is pooled from 2-4 independent experiments and is shown as mean +/- SEM, analyzed by Mann-Whitney U-tests or two-tailed unpaired t-tests. *P<0.05, **P<0.01. d, Schematic depiction of the Candidalysin-IL-1β-CXCL1 protective immune axis acting via Card9-expressing microglia to mediate protective neutrophil accumulation to the brain following invasive C. albicans infection of the CNS.

**Supplementary Fig. 1:** Microglia express CARD9-coupled C-type lectin-receptors.

Microglia were MACS-sorted from uninfected wild-type brains and analyzed for expression of
the indicated C-type lectin receptors (and Card9 as control) by qRT-PCR, relative to Gapdh.

Each point represents pooled microglia from an individual mouse.

**Supplementary Fig. 2: Gating strategy used to define myeloid cells in the C. albicans-infected brain.** Gates and their associated frequency are outlined in blue. Titles in blue above each plot denote the cell population being defined that plot.

**Supplementary Fig. 3: Optimal neutrophil phagocytosis of Candida requires Dectin-1 and Dectin-2 in the C. albicans-infected brain.** Dectin-1 and Dectin-2-deficient mice and their wild-type controls were infected with 5x10^6 GFP-expressing C. albicans and brain leukocytes analyzed 2 hours later by flow cytometry, using the gating strategy shown in Fig S2. The percentage of GFP^+ neutrophils was used as a measure of in vivo phagocytosis. Each point represents an individual mouse. Data is pooled from 1-3 experiments and shown as mean +/- SEM. Histograms are gated on live CD45^+Ly6G^+CD11b^+ neutrophils. *P<0.05, by unpaired two-tailed t-test.

**Supplementary Fig. 4: Chemoattractant receptors LTB4R1 and FPR1 are not required for protective neutrophil recruitment to the fungal-infected brain.** Animals deficient in chemoattractant receptors were infected and analyzed as described for Fig. 1 (n=6-15) for (a) neutrophil recruitment to the brain at 24h, and (b) fungal burdens at 72h post-infection. 'Wild type' refers to appropriate matched control animals for each knock-out line for gender, age and genetic background. Individual points represent different mice. Data is pooled from 2-3
independent experiments and shown as mean +/- SEM, and analyzed by unpaired two-tailed t-test or Mann Whitney U-test. **P<0.01, ****P<0.001.

Supplementary Fig. 5: Abundance and activation markers of Card9-/- microglia. The number of microglia from Card9+/+ and Card9-/- mice were determined at the steady-state and during infection at the indicated time points (left). Expression levels of lineage and activation markers were determined in 8-10-week-old uninfected mice by flow cytometry (right). Data shown is pooled from 2-3 independent experiments.

Supplementary Fig. 6: Microglia and Ly6C^{hi} monocytes were FACS-sorted from 24h infected brains from Card9^{fl/fl}Cx3cr1^{CreER/-} and Card9^{fl/fl}Cx3cr1^{CreER+/+} littersmates that had been tamoxifen-pulsed 4 weeks earlier. Cells were pooled from 4-5 brains per group, and Card9 expression quantified by qRT-PCR relative to Gapdh. Data is pooled from 2 independent sorting experiments.
Figure 1

(a) Hours post-infection brain CFU/g

(b) 24 hours post-infection

(c) Dectin-1, Dectin-2, Dectin-3, Mincle, Dectin-1/FcRγ

(d) Malt1+/+, Malt1−/−
Figure 2

24 hours post-infection

a) Neutrophils/brain (x10^4)

- IL-1R
- MyD88
- IL-1α
- IL-1β
- IL-1α/IL-1β

b) Brain CFU/g

- <10^1
- 10^0
- 10^1
- 10^2
- 10^3
- 10^4
- 10^5
- 10^6
- 10^7

- 24 hours post-infection

- 72 hours post-infection

- Myd88^-/-
- Il1r^-/-
- Il1a^-/-
- Il1b^-/-
- Il1a^-/-Il1b^-/-

24 hours post-infection

- CCR1
- CXCR1
- CXCR2
- CXCL1

- Cxcr1^-/-
- Ccr1^-/-
- Cxcr2^-/-
- Cxcl1^-/-

- hours post-infection
Figure 3

a) IL-1β and CXCL1 levels in wild type, Cxcl1/−, and Il1b/− mice. Wild type > Cxcl1/− > Il1b/−.

b) Proportion of total CD45+ cells and proportion of cytokine+ cells (% of CD45+) over 24 hours post-infection. CD45+CD11b+ cells are increased in the absence of CXCL1.

c) Frequency of CXCL1+ cells and proportion of total CD45+ cells (% for each cell type) in wild type, Il1b/−, and Cxcl1/− mice. Ly6Chigh+ monocytes and neutrophils are increased in the absence of CXCL1.
Figure 4

**a** 24 hours post-infection

![Graph showing brain CFU/g and neutrophils/brain](image)

- BWP17: \( hgc1\Delta\Delta \) + HGC1
- \( hgc1\Delta\Delta \)

**b** 24 hours

- brain CFU/g
- neutrophils/brain \( \times 10^5 \)

**C** 24 hours post-infection

- neutrophils/brain \( \times 10^5 \)

**d** 24 hours post-infection

- IL-1β (pg/mL)
- C-XCL1 (ng/mL)

**e** 24 hours post-infection

- brain CFU/g
- neutrophils/brain \( \times 10^5 \)

Additional notes:

- CAI4 + Clp10
- \( hgc1\Delta\Delta \) + Clp10
Figure 5

(a) Microglia

Frequency pro-IL-1β+ (%)

Ly6C^hi monocytes

Frequency CXCL1^+ (%)

(b) Microglia

Frequency pro-IL-1β+ (%)

Ly6C^hi monocytes

Frequency CXCL1^+ (%)

Neutrophils

Frequency pro-IL-1β+ (%)

Neutrophils

Frequency CXCL1^+ (%)

BWP17 (ll1b^−/−) BWP17 (wild type) ece1^−/− (wild type)

BWP17 (Cxcl1^−/−) BWP17 (wild type) ece1^−/− (wild type)
Figure 6

**a**

- **IL-1β (pg/mL)**
- **Fold change**
- **Candidalysin (µM)**

**b**

- **LDH release**
- **Fold change**
- **Candidalysin (µM)**

**c**

- **microglia/astrocyte co-cultures**
- **ELISA co-culture supernatants**
- **CXCL1 (ng/mL)**
- **Candidalysin (µM)**

**d**

- **intracellular FACS**
- **CXCL1+ microglia (%)**
- **Candidalysin (µM)**
- **Astrocytes**

**e**

- **cFos**
- **pMKP1/2**
- **β-actin**
- **Candidalysin (µM)**
- **LPS**

**f**

- **p38 inhibition**
- **IL-1β (%) of control**
- **cFos inhibition**
- **SB203580 (µM)**
- **T-5224 (µM)**
Figure 7

(a) Frequency of positive CD45+ cells (%)

(b) Frequency of positive microglia (normalized to Card9+/-)

(c) Relative expression of i1b

(d) Pro-IL-1β/β-actin ratio (normalized to WT)

(e) NLRP3/β-actin ratio (normalized to WT)

(f) NLRP3 KO:
  - Neutrophils/brain (x10^5)
  - Brain fungal burdens (CFU/g)
Figure 8

a) Fungal burdens (CFU/g) in the brain and kidney 24 and 72 hours post-infection.

b) Neutrophils in the brain (×10^4) per brain.

C) Frequency of positive microglia (normalized to CreER−) after stimulation with zymosan or LPS.

D) Diagram showing Candidalysin expression by C. albicans hyphae leading to fungal clearance, IL-1β secretion by microglia, and CXCL1 expression by astrocytes.

Legend:
- Card9^{fl/fl} Cx3cr1^{CreER−/−}
- Card9^{fl/fl} Cx3cr1^{CreER+/−}
- p38
- cFos
- CARD9^{+} microglia
- CXCL1
- astrocyte
- fungal clearance

Statistical significance:
- * p < 0.05
- ** p < 0.01

Data presentation includes scatter plots with statistical comparisons.
Figure S1

relative expression

Figure S2

leukocytes

singlets

live/dead (Indo-1-violet)

live cells

CD45+ cells

Ly6C<sup>hi</sup> monocytes

neutrophils

microglia

leukocytes singlets

live/dead (Indo-1-violet)

live cells

CD45+ cells

Ly6C<sup>hi</sup> monocytes

neutrophils

microglia
Figure S3

GFP-Candida neutrophils (%)

GFP-Candida

24 hours post-infection

<10^4

10^5

GFP-Candida neutrophils

wild type

Clec7a^+/

Clec4n^+/

Figure S4

24 hours post-infection

neutrophils/brain (x10^4)

Leukotriene B4 Receptor 1

fMet-Phe-Leu Receptor 1

brain CFU/g

hours post-infection

24 72

<10^1

10^2

10^3

10^4

10^5